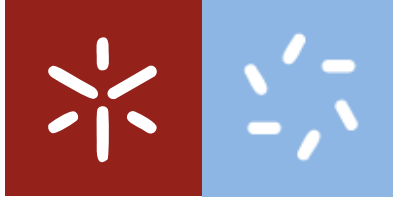


**Universidade do Minho**  
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Fábio José dos Santos Trigo

**The physiology and biochemistry of  
Methylacidiphilum fumariolicum SolV**





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**The physiology and biochemistry of  
Methyacidiphilum fumariolicum SolV**

Master Thesis

Master Degree in Molecular Genetics

Work conducted under the supervision of

**Dr. Huub Op den Camp**

**and**

**Professora Doutora Ana Preto**

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## ABSTRACT

“*Methyloacidiphilum fumariolicum*” SolV is a verrucomicrobial methanotroph that can grow in extremely acidic environments at high temperature. In this study the nitrosative stress on strain SolV was investigated using batch cultivations. Nitrite production was measured at different ammonium concentrations under methane concentrations in a range of 0 to 3%. In this study we found that SolV can use ammonium when methane is limited. The second part of this study is the growth of this strain SolV on higher alkanes such as ethane, propane and butane. Strain SolV has three *pmoCAB* operons and one of these operons was not expressed while growing on methane. Therefore, it was hypothesized that the third operon might be expressed under growth conditions in the presence of higher alkanes. Thus, studies on higher alkanes (ethane and propane) were performed. Strain SolV can grow on both alkanes but further experiments and optimizations are necessary.

## RESUMO

“*Methylophilum fumariolicum*” SolV é um metanotrofo verrucomicrobiano que cresce em ambientes extremamente ácidos a uma temperatura elevada. Neste estudo o nitrossativo stress na estirpe SolV foi investigada usando cultivações batch. A produção de nitrito foi medida em diferentes concentrações de amónio sob diferentes concentrações de metano numa gama de 0 a 3%. Neste estudo descobrimos que SolV pode consumir amónio quando metano é limitado.

A segunda parte deste estudo é o crescimento desta estirpe SolV em alcanos superiores tais como etano, propano e butano. A estirpe SolV tem três operões *pmoCAB* e um destes operões não foi expresso durante o crescimento em metano. Portanto, foi hipotetizado que o terceiro operão poderia ser expresso sob condições de crescimento na presença de alcanos superiores. Assim, estudos em alcanos superiores (etano e propano) foram realizados. A estirpe SolV cresce em ambos os alcanos mas futuras experiências e optimizações são necessárias.

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## LIST OF ABBREVIATIONS

• CH <sub>4</sub>	Methane
• OH·	Hydroxyl radical
• NO <sub>x</sub>	Nitrogen oxides
• CH <sub>2</sub> O	Formaldehyde
• O <sub>3</sub>	Ozone
• CO	Carbon monoxide
• N-damo	nitrite-driven anaerobic methane oxidation
• O <sub>2</sub>	Oxygen
• RuMP	ribulose monophosphate pathway
• RuBisCO	1,5 bisphosphate carboxylase/oxygenase
• CH <sub>3</sub> OH	Methanol
• H <sub>2</sub> O	Water
• CHOOH	Formate
• NAD	Nicotinamide adenine dinucleotide
• ATP	Adenosine-5'-triphosphate
• FDH	Formate dehydrogenase
• MMO	Methane monooxygenase
• AMO	Ammonia monooxygenase
• pMMO	Particulate methane monooxygenase
• sMMO	Soluble methane monooxygenase
• MDH	Methanol dehydrogenase
• FADH	Formaldehyde dehydrogenase
• CO <sub>2</sub>	Carbon dioxide
• CBB	Calvin-Benson-Bassham
• N <sub>2</sub>	Nitrogen
• ICP-MS	Inductively coupled plasma mass spectrometry
• CH <sub>3</sub> SH	Methanethiol
• NH <sub>4</sub> <sup>+</sup>	Ammonium
• NH <sub>2</sub> OH	Hydroxylamine
• NO <sub>2</sub> <sup>-</sup>	Nitrite

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# Introduction

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# 1-INTRODUCTION

## 1.1-Methane

Methane (CH<sub>4</sub>) is a very important fossil fuel for both households and industry. Methane is known as the second most important greenhouse gas and it contributes up to 20 % of the greenhouse effect. In addition, methane is approximately 30 times more effective than CO<sub>2</sub> as a greenhouse gas on a 100 year-scale indicating the importance of its study as a powerful climate affecting gas in the atmosphere (Forster *et al.*, 2007; Houghton *et al.*, 1996; Denman *et al.*, 2007; Shindell *et al.*, 2009).

The concentration of methane in the atmosphere has been greatly increased over the last 200 years due to the activity of mankind. Recently the report of the Intergovernmental Panel on Climate Change indicates that the methane concentration in atmosphere has been decreased during the last 5 years (Singh, 2011). It is not known if the decrease of the methane in the atmosphere is caused by a decline in emission or an increase in methane sink activities.

The biogenic methane produced by Archaea under anoxic conditions during decomposition of organic matter is up to 80% and it is emitted from anthropogenic activities (landfills, rice paddy fields and coal mining) and natural ecosystems (ruminants, wetlands and termites) (Conrad, 2009; Etiope *et al.*, 2011; Schink, 1997; Thauer, 1998). Due to the global warming concern, it is important to study the sinks and sources of methane.

The remaining part of the methane emission comes from thermal decomposition of organic matter (> 80 °C) within the Earth's crust by which methane is expelled to the atmosphere from geothermal areas such as mud volcanoes, mud pots, fumaroles and seeps. (Conrad, 2009; Etiope & Klusman, 2002; Etiope *et al.*, 2011).

These environments add up to 70 Tg methane emission per year in a total of 600 Tg per year (Castaldi & Tedesco, 2005; Kvenvolden & Rogers, 2005), though these emissions need to be quantified more accurately (Etiope & Klusman, 2002). All methane does not reach the atmosphere, because a large part is oxidized by anaerobic and aerobic methane-oxidizing bacteria which are called methanotrophs. These microorganisms are the central engine to keep the methane balance on the earth. The

methane that escapes from the oxidation of methanotrophs is oxidized in the troposphere by the hydroxyl radical ( $\text{OH}^\cdot$ ) that is the major radical in this layer of the atmosphere forming water vapor and carbon dioxide. In the presence of high levels of nitrogen oxides ( $\text{NO}_x$ ), methane oxidation with the hydroxyl radical leads to the formation of formaldehyde ( $\text{CH}_2\text{O}$ ), ozone ( $\text{O}_3$ ) and carbon monoxide ( $\text{CO}$ ). Through this reaction the removal of methane is around 490 Tg per year. Thus the hydroxyl radical is the most important factor that removes methane from the atmosphere (Houweling *et al.*, 1999; Khalil & Shearer, 2000; Lelieveld *et al.*, 1998; Moss *et al.*, 2000).

Recent studies showed that volcanoes act as important methane sinks by the activity of aerobic verrucomicrobial methanotrophs and their methane oxidation. They were isolated independently from volcanic regions in Russia, New Zealand and Italy (Dunfield *et al.*, 2007; Islam *et al.*, 2008; Pol *et al.*, 2007). In this study, we concentrate on *Methylococcoides burtonii* SolV the recently isolated aerobic methanotroph from a volcanic region near Naples in Italy (Pol *et al.* 2007).

## **1.2-Methanotrophs**

### **1.2.1-Anaerobic methanotrophs**

Methane-oxidizing archaea and sulfate-reducing bacteria together perform anaerobic oxidation of methane (Boetius *et al.*, 2000; Valentine & Reeburgh, 2000). Furthermore, this process can be performed by nitrite-reducing bacteria (Ettwig *et al.*, 2008; Raghoebarsing *et al.*, 2006).

Despite evidence for sulfate-driven methane oxidation from profile analysis in anoxic organic rich sediments (Barnes & Goldberg, 1976; Martens & Berner, 1974; Reeburgh, 1976) the process was controversial for some time because neither responsible microorganisms nor mechanisms could be identified. It has been shown that sulfate-reducing bacteria and anaerobic methanotrophic archaea perform this process in deep sea environments (Boetius *et al.*, 2000).

First report on nitrite-driven anaerobic methane oxidation (n-damo) hypothesized that a consortium of archaea conducting reverse methanogenesis was

affiliated with a denitrifying bacterial partner (Raghoebarsing *et al.*, 2006). Thereafter, it has been shown that the bacterial partner could complete the process independently (Ettwig *et al.*, 2008), and the bacterium responsible for the n-damo process belongs to the division 'NC10' (Rappe & Giovannoni, 2003).

In anoxic environments the n-damo process follows a classical aerobic methane oxidation pathway (Ettwig *et al.*, 2010). It has been shown that '*Candidatus* Methyloirabilis oxyfera' has a unique ability to produce intracellular oxygen through a denitrification pathway. Geochemical evidences exist for iron (III)-driven anaerobic methane oxidation as a thermodynamically favorable process (Beal *et al.*, 2009; Sivan *et al.*, 2007; Zehnder & Brock, 1980). Recent geochemical studies indicate that this process occurs in deep ocean sediments being located below a depth of 25-cm, which is deeper than the location where sulfate and nitrate are available as well as the zone of methanogenesis (Sivan *et al.*, 2011).

### **1.2.2-Aerobic methanotrophs**

The aerobic methanotrophs among the methylotrophs form a unique group of microorganisms, because they utilize methane as the only source of carbon and energy (Hanson & Hanson, 1996). Thus far 18 genera of aerobic methanotrophs have been described within the bacterial phylum *Proteobacteria* and these proteobacterial methanotrophs are divided into two subphyla, the Gamma and Alpha *proteobacteria*.

The methanotrophic members of the *Gammaproteobacteria* (type I methanotrophs) are represented by the family *Methylococcaeae* and the methanotrophic members of the *Alphaproteobacteria* (type II methanotrophs) are represented by two families, the *Methylocystaceae* and the *Beijerinckiaceae*.

The type I and II of methanotrophs are categorized based on their internal membrane structure (ultrastructure), phylogeny (*Gammaproteobacteria* versus *Alphaproteobacteria*), cell morphology, the dominant phospholipid fatty acids (18C versus 16C) and the metabolic pathways used for biomass production (serine pathway versus ribulose monophosphate (RuMP) pathway (Chistoserdova, 2011; Chistoserdova *et al.*, 2009; Hanson & Hanson, 1996). The terms type I and II methanotrophs are now synonyms of *Gammaproteobacteria* and *Alphaproteobacteria*. (Op den Camp *et al.*, 2009). The family *Methylococcaceae* of the *Gammaproteobacteria* contains the most genera of the proteobacterial methanotrophs, but not all the genera fit to this family, for



example *Methylohalobius* and *Methylothermus* are classified in this family but analysis of the 16S ribosomal RNA and *pmoA* genes demonstrate that these microorganisms may not be monophyletic with this family (Heyer *et al.*, 2005; Tsubota *et al.*, 2005).

Another example is the family *Crenotrichaceae* (*Crenothrix* and *Clonothrix*), which is validated but it is phylogenetically a subset of the *Methylococcaceae* (Op den Camp *et al.*, 2009; Stoecker *et al.*, 2006; Vigliotta *et al.*, 2007).

Aerobic proteobacterial methanotrophs are microorganisms that are widespread in nature and man-made environments such as marine and fresh waters, sediments, landfills, rice paddies and soils where they consume up to 90% of the methane produced by methanogenic archaea in the anoxic zones of these environments after the organic matter is degraded anaerobically (Segers, 1998).

These microorganisms are also found in symbiosis with marine invertebrates (the sponge *Cladorhiza methanophila*, the hydrothermal vent snails tubeworms of the *Siboglinum* genus, *Ifremeria nautilei* and *Alviniconcha hessleri*, and deep-sea bathymodiolin mussels of two genera, *Idas* and *Bathymodiolus*) at hydrothermal vents and cold fonts in the deep Sea (Petersen & Dubilier, 2009).

Physiological experiments using labeled methane in whole animals or tissues containing the methanotrophic symbionts, 16S ribosomal RNA analysis, activity assays with crucial enzymes of the methane oxidation pathway and ultrastructure analysis of the internal cytoplasmic membrane system are various methods used to demonstrate that symbionts are methanotrophs.

Thus far, only type I methanotrophs appear to be involved in the symbiosis with marine invertebrates and that may be caused by the efficiency of the RuMP pathway used for carbon assimilation. On the other hand the serine pathway used by type II methanotrophs is less efficient (Leak *et al.*, 1985).

Marine invertebrate fully rely on their symbiont for most or even all their carbon and energy needs, so it is advantageous for them to be in association with type I methanotrophs.

Type II methanotrophs are found in association with wetland plants (Raghoebarsing *et al.*, 2005). These plants are autotrophs. Thus these associations with type II methanotrophs supply plants only around 15% of the cellular carbon, because the dependence for their organic carbon is much lower.

### 1.3-The discovery of the aerobic verrucomicrobial methanotrophs

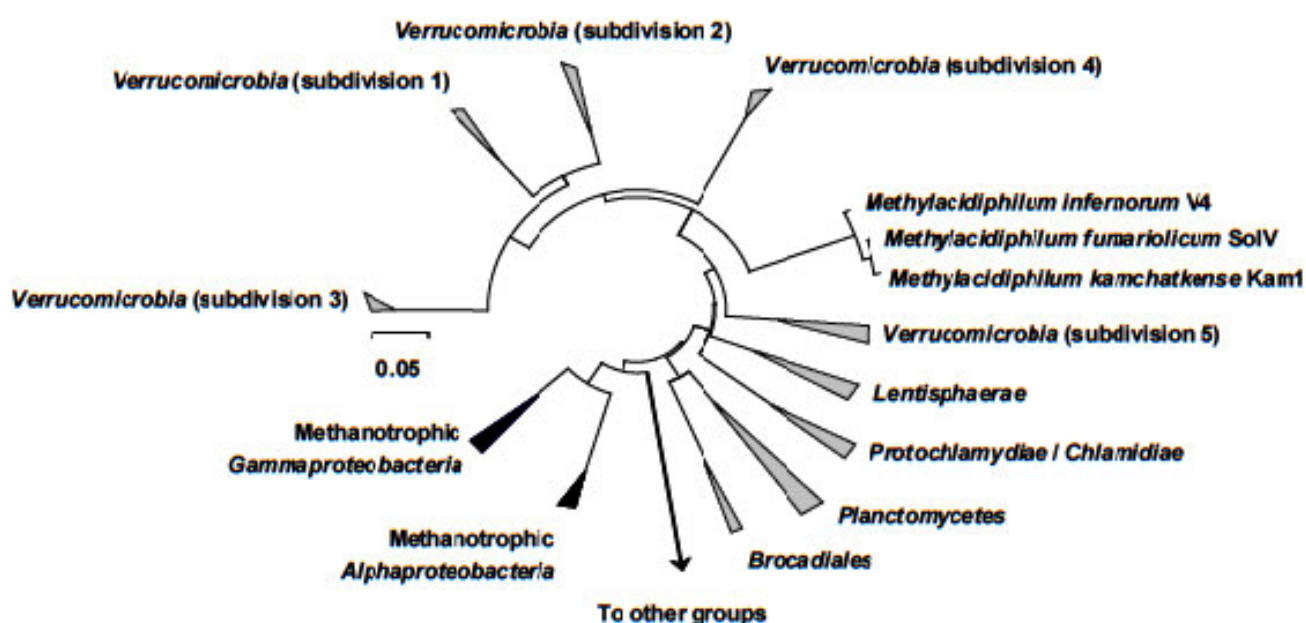
Mesophils and neutrophils are the most known genera within the aerobic proteobacterial methanotrophs and the only exceptions are the *Methylothermus*, *Methylococcus* and *Methylocaldum* genera that are found in geothermal springs where the optimal growth temperatures were reported in a range of 45-58°C.

There are reports on *Methylocystis*, *Methylocapsa*, *Methylocella* and *Methyloferula* which are mild acidophilic genera that are abundant in peatlands, and these microorganisms grow at pH between 3.5 to 7.5 (Dedysh *et al.*, 2000; Dedysh *et al.*, 2002; Dedysh *et al.*, 2007; Trotsenko & Khmelenina, 2002; Tsubota *et al.*, 2005; Vorobev *et al.*, 2011). A report of geothermal areas shows that a significant amount of methane is consumed in areas with high temperatures (50-95°C) and very low pH (below 1.8) (Castaldi & Tedesco, 2005), which indicates methanotrophy under more extreme conditions.

In late 2007 to early 2008, this geological evidence was supported and validated by three independent isolations of novel aerobic methane oxidizing bacteria in pure cultures from volcanic regions (Dunfield *et al.*, 2007; Islam *et al.*, 2008; Pol *et al.*, 2007). These three cultures were isolated from the Hell's Gate, Tikitere (New Zealand), the Uzon Caldera, Kamchatka, (Russia) and Solfatara at Pozzuoli near Naples (Italy).

Based on 16S ribosomal RNA gene sequences, all the three strains isolates (V4, Kam1, and SolV) could be identified as members of the *Verrucomicrobia* phylum and they belong to a single genus for which the name *Methylacidiphilum* was proposed (Op den Camp *et al.*, 2009; Fig.1).

Environmental clone libraries show a large biodiversity in *Verrucomicrobia* and their presence in many ecosystems (landfill leachate, acid rock drainage, soils and peat bogs), often in relative high numbers and most members remain uncultivated and their physiology is not well understood (Wagner & Horn, 2006). This was the first time that the widely distributed *Verrucomicrobia* phylum was associated to a geochemical cycle.



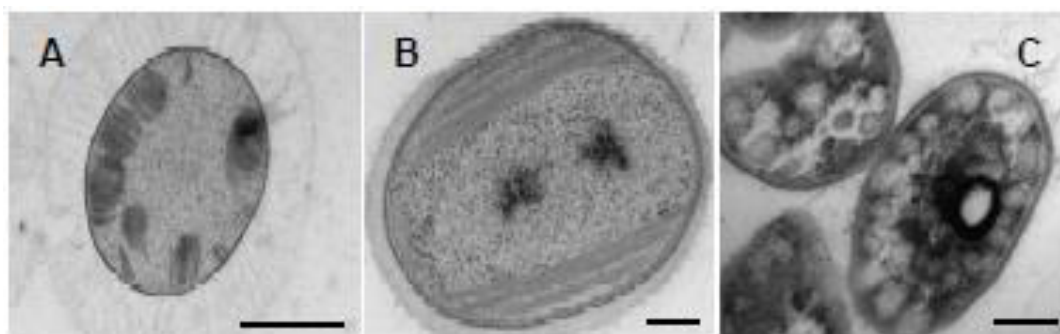
**Figure 1** - Phylogenetic tree showing the position of the verrucomicrobial aerobic methanotrophs (strains V4, SolV and Kam1) relative to the proteobacterial aerobic methanotrophs (modified from Op den Camp *et al.* (2009)).

A complete genome sequence was published for *Methyloacidiphilum infernorum* strain V4 (Hou *et al.*, 2008) and a draft genome was available for *Methyloacidiphilum fumariolicum* strain SolV (Op den Camp *et al.*, 2009; Pol *et al.*, 2007) and this was added to the several verrucomicrobial genome assemblies (van Passel *et al.*, 2011).

Preliminary studies of *Methyloacidiphilum* strains (V4, SolV and Kam1) indicated some similarities but also differences with the proteobacterial aerobic methanotrophs such as carbon dioxide fixation pathways and distinct enzymes of the methane oxidation.

It was observed that *Methyloacidiphilum* strain has circular bodies of 50-70 nm (Fig.2) instead of stacked membrane structures characteristics for methanotrophs

expressing particulate methane monooxygenase. Afterwards, it was hypothesized that these circular bodies in *Methylococcoides* strain may be compared with carboxysomes or novel subcellular compartments for methane oxidation (Islam *et al.*, 2008; Op den Camp *et al.*, 2009). These carboxysomes are compartments that are found in cyanobacteria and in a limited numbers of chemoautotrophs and thought to enhance carbon fixation by increasing its level for the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which has a low affinity for carbon dioxide (Yeates *et al.*, 2008).



**Figure 2** - (A) Transmission electron micrograph of type I (scale bar, 1  $\mu\text{m}$ ) and (B) type II (scale bar, 200 nm) proteobacterial aerobic methanotrophs, showing the discshaped and the parallel membranes in these microorganisms, respectively. (C) In *Methylococcoides fumariolicum* strain SolV circular bodies of about 50-70 nm were observed (scale bar, 200 nm). Pictures were modified from Kip *et al.* (2011) and Pol *et al.* (2007).

## 1.4 - proteobacterial vs. verrucomicrobial methanotrophs

### 1.4.1 - Energy metabolism in aerobic methanotrophs

The chemical equation of  $\text{CH}_4 + 2 \text{O}_2 \rightarrow \text{CO}_2 + 2 \text{H}_2\text{O}$  indicates the aerobic oxidation of methane. During this oxidation, energy is obtained at the level of methanol ( $\text{CH}_3\text{OH}$ ), formaldehyde ( $\text{CH}_2\text{O}$ ) and formate ( $\text{CHOOH}$ ) oxidation (Fig. 3) (Chistoserdova *et al.*, 2009; Hanson & Hanson, 1996).

## 1.4.2- Methane oxidation pathway

### 1.4.2.1-Methane oxidation

The first step in the methane oxidation pathway is the conversion of methane to methanol by methane monooxygenase (MMO). It is known that Proteobacterial aerobic methanotrophs have two distinct forms of this enzyme; the particulate membrane-associated form (pMMO, cytochrome *c* dependent) and the soluble cytoplasmic form (sMMO, NADH-dependent; figs.3B and 3A; Hanson & Hanson, 1996). The sMMO expressed under conditions of copper limitation is only found in a certain number of methanotrophs compared to pMMO that is expressed under conditions of no copper limitation and it is present in all known methanotrophs, but *Methylocella vestris* BL2 (Chistoserdova, 2011).

It is known that proteobacterial aerobic methanotrophs contain several copies of *pmo* operons. Two similar copies of *pmoCAB1* in both type I and type II proteobacterial methanotrophs were found (Murrell *et al.*, 2000; Semrau *et al.*, 1995). The hypothesis is that the sequence-identical copies have appeared by gene insertions and duplications. By mutation studies in *Methylococcus capsulatus* Bath the requirement of both sequence-identical copies of pMMO was shown (Stolyar *et al.*, 1999).

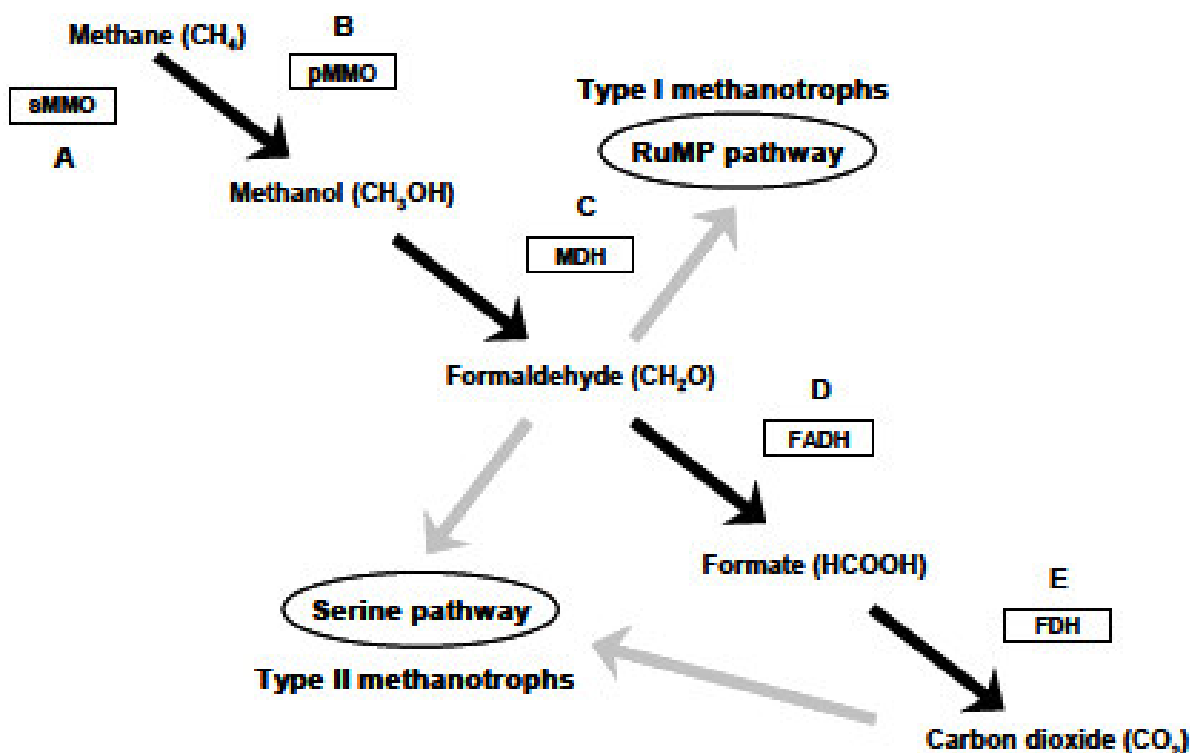
The *pmoCAB2* was demonstrated to be absent in type I proteobacterial methanotrophs, and widely but not universally distributed in type II proteobacterial methanotrophs (Baani & Liesack, 2008).

Lately, it was discovered that the genera of type I proteobacterial methanotrophs encode a new sequence-divergent *pmo*. Unlike the CAB order for pMMO and AMO operon, it was encoded in the *pxmABC* operon (Tavormina *et al.*, 2011). The presence of sequence-divergent copies supports the hypothesis that these enzymes evolved from the same ancestor but took different physiological function on different environmental conditions.

Using the complete genome of *M. infernorum* strain V4 and the draft genome of *M. fumariolicum* strain SolV it was shown that none of the genes encoding the sMMO subunits were found in these verrucomicrobial aerobic methanotrophs despite of three complete *pmoCAB* operons and an extra copy of *pmoC* being identified in these strains (Hou *et al.*, 2008; Op den Camp *et al.*, 2009; Pol *et al.*, 2007).

The analysis of the draft genome of *M. kamchatkense* strain Kam1 showed orthologues of each of the three *pmo* operons discovered in strains V4 and SolV (Op den Camp *et al.*, 2009).

A high similarity between the genes *pmoA1*, *pmoA2* and *pmoA3* of each strain and the *pmoA4* of Kam1 was found, based on phylogenetic analysis of the Verrucomicrobial *pmoA* genes, so they represented a new branch, which is distinct from *pmoA* and *amoA* genes of other cultured organisms (Op de Camp *et al.*, 2009).



**Figure 3** - Established pathways for the oxidation of methane (black arrows) and assimilation of formaldehyde (grey arrows) in type I and type II proteobacterial aerobic methanotrophs (modified from Hanson & Hanson, 1996). The enzymes involved in methane oxidation are indicated within the boxes. (A) The soluble methane monooxygenase (sMMO). (B) The particulate methane monooxygenase (pMMO). (C) The methanol dehydrogenase (MDH). (D) The formaldehyde dehydrogenase (FADH). (E) The formate dehydrogenase (FDH). The formaldehyde, produced during the oxidation of methane can be assimilated via the serine pathway or the ribulose monophosphate (RuMP) pathway (adapted from khadem PhD thesis).

#### 1.4.2.2-Methanol oxidation

The second step of methane oxidation pathway of proteobacterial aerobic methanotrophs is the oxidation of methanol to formaldehyde by the methanol

dehydrogenase (MDH) enzyme (Fig.3C). The MDH consists of large and small subunits, encoded by the *mxoFI* genes, and requires a cytochrome c electron acceptor and a cofactor (pyrroloquinoline quinone). The later encoded by *mxoG* gene and it also needs calcium insertion to its active center.

Recently, the activity of this enzyme in verrucomicrobial methanotroph *M. fumariolicum* strain SolV was shown to be dependent (Pol *et al.*, 2007; 2013) on lanthanides, a group of rare earth elements (cerium, lanthanum, neodymium and praseodymium) as a cofactor in a homodimeric MDH.

#### **1.4.2.3-Formaldehyde oxidation**

The third step of the methane oxidation pathway is the conversion of formaldehyde into formate by the formaldehyde dehydrogenase enzyme (Fig.3D). This step is very important for methanotrophs because it keeps intracellular formaldehyde concentrations at non-toxic levels and it is extremely important for energy generation (Chistoserdova, 2011).

#### **1.4.2.4-Formate oxidation**

The fourth and last step in methane oxidation pathway is the conversion of formate into carbon dioxide and it is performed by the enzyme formate dehydrogenase (FDH) (Fig. 3E).

It is known that different organisms as well as Methylophs have different types of formate dehydrogenases. A good example is *M.extorquens* species which contains four different FDH enzymes: a novel type of FDH, a predicted molybdenum-containing FDH, a predicted cytochrome-linked FDH that is likely periplasmic and a tungsten-containing FDH (Chistoserdova, 2011).

Based on the genome data of the strains V4 and SolV (verrucomicrobial methanotrophs), it was suggested that NAD-dependent formate dehydrogenase enzyme conducts this last step of methane oxidation pathway (Hou *et al.*, 2008; Pol *et al.*, 2007).

#### 1.4.2.5-Carbon assimilation

In this pathway (Figure 3) carbon assimilation is located at the oxidation level of formaldehyde in aerobic methanotrophs (Hanson & Hanson, 1996).

The group of *Alphaproteobacteria* uses the serine pathway while the group of *Gammaproteobacteria* uses the RuMP pathway for the assimilation of formaldehyde to produce CO<sub>2</sub> (Chistoserdova *et al.*, 2009).

Genome data of non-proteobacterial aerobic methanotrophs (*Methylacidiphilum infernorum* V4, *Methylacidiphilum fumariolicum* SolV and ‘*Candidatus Methylomirabilis oxyfera*’ [Ettwig *et al.*, 2010; Hou *et al.*, 2008; Op den Camp *et al.*, 2009]) and proteobacterial methanotrophs (*Methylococcus capsulatus* Bath, *Methylocella silvestris* BL2 [Chen *et al.*, 2010; Ward *et al.*, 2004]) showed the presence of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), Calvin-Benson-Bassham (CBB) cycle key enzyme. It was found that *M. capsulatus* Bath contained RuBisCO in an active form (Stanley & Dalton, 1982), and the genome analysis suggested that a variant of the CBB cycle might be present (Kelly *et al.*, 2005; Ward *et al.*, 2004).

Analyses of the draft genome of *M. fumariolicum* strain SolV showed that this verrucomicrobial methanotrophs and a complete genome sequence of *M. infernorum* strain V4 (Hou *et al.*, 2008) lack the key enzymes for both the ribulose monophosphate and serine pathways (Op den Camp *et al.*, 2009), but it was shown that a complete set of genes encoding the enzymes of the CBB cycle is present which indicates that these methanotrophs could fix CO<sub>2</sub>, presumably using CH<sub>4</sub> as an energy source.

The Calvin-Benson-Bassham cycle has been related with a large use of ATP per mol of CO<sub>2</sub> fixed and was never thought to be considered a pathway that supports growth on CH<sub>4</sub> (Chistoserdova *et al.*, 2009).

Khadem *et al.*, (2011) used <sup>13</sup>CH<sub>4</sub> or <sup>13</sup>CO<sub>2</sub> in growth experiments to show that CO<sub>2</sub> is the only carbon source for *M. fumariolicum* strain SolV during growth on CH<sub>4</sub>. Similarly in this paper it was demonstrated that all genes needed for a complete CBB cycle were transcribed, using the transcriptome study of the SolV. It was also shown that the small and the large subunits of RuBisCO were highly expressed.



## 1.5-Objectives

The aim of this project was the study of the biochemistry and physiology of the verrucomicrobia *methyladiciphilum fumariolicum* strain SolV.

The project is divided in two parts:

In the first part, we studied how strain SolV could handle nitrosative stress which is the result of  $\text{NH}_4^+$  oxidation to  $\text{NH}_2\text{OH}$  and further  $\text{NO}_2^-$  in the presence of various concentrations of  $\text{CH}_4$ . The pMMO enzyme involved in the first step of methane oxidation can also oxidize ammonium to hydroxylamine which is a highly toxic compound, and it further be converted to nitrite which is also toxic. Thus, strain SolV should use a mechanism to detoxify these compounds.

The second part of this project was the study of growth in the presence of higher alkanes including ethane, propane and butane. Strain SolV has three *pmoCAB* operons and one of these operons was not expressed under nitrogen fixing and oxygen limited cultures, where electron donor was methane (Khadem *et al.*, 2012). It is hypothesized that the third operon might be expressed in growth conditions using higher alkanes.

## **Materials & Methods**

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## **2-MATERIALS & METHODS**

### **2.1-Microorganism**

In this study, we used the bacteria *Methylophilum fumariolicum* strain SolV, which was originally isolated from the volcanic region, Campi Flegrei, near Naples, Italy (Pol *et al.*, 2007).

### **2.2-Medium composition for growth**

The medium used in this study to grow strain SolV contained in g/L:  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.0406;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.03;  $\text{Na}_2\text{SO}_4$ , 0.142;  $\text{K}_2\text{SO}_4$ , 0.35;  $(\text{NH}_4)_2\text{SO}_4$ , 0.528 and the concentration of trace elements were 0.1  $\mu\text{M}$  (Ni, Co, Mo, Zn, Ce); 2  $\mu\text{M}$  (Mn, Fe); 3  $\mu\text{M}$  (Cu). The pH of medium was brought to a value of 2.7 with  $\text{H}_2\text{SO}_4$  before autoclaving.  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  was autoclaved separately and added to avoid the formation of precipitation. The amounts were calculated based on the growth till optical density (OD) 1, unless otherwise stated.

### **2.3-Ammonium determination**

Ammonium concentrations were measured using the ortho-phthaldialdehyde (OPA) method (Taylor *et al.*, 1974). Samples were centrifuged 5 minutes at 13000 rpm and diluted 5 times prior to the determination. The standard curves were made using concentrations of ammonium in a range of 0 till 5 mM ammonium instead of samples and then following the protocol.

### **2.4-Nitrite determination**

Nitrite concentrations were measured using sulfanilic acid (Reagent A) and naphthylethylene diaminedihydrochloride (Reagent B) method (Griess, 1879). Samples were centrifuged 5 minutes at 13000 rpm and based on the expected nitrite concentrations, diluted or undiluted samples were used in this protocol. The standard

curves were made using concentrations of nitrite in a range of 0 till 0.1 mM nitrite instead of samples and then following the protocol.

## **2.5-Gas analyses**

Methane, ethane and propane (100  $\mu$ l) were analyzed on a HP 5890 gas chromatograph (GC) (Agilent, USA) equipped with a Porapak Q column (1.8 m x 2 mm) and a flame ionization detector.

## **2.6-Batch cultivation**

Batch incubations were prepared with 60 ml serum bottles containing 5ml culture from reactor that were washed twice with medium and sealed with grey stoppers. Incubations were performed in duplicate at 55°C with shaking at 380 r.p.m. The headspace contained air as the oxygen source and CH<sub>4</sub> and CO<sub>2</sub> concentrations of 10 and 5 %, respectively. In incubations to test nitrite production, CH<sub>4</sub> and ammonium concentrations were used in a range of 0-3 % and 0.5-16 mM, respectively. Nitrite and ammonium were measured using the nitrite and ammonium determination protocols.

The growth on higher alkanes (ethane and propane) was also studied using batch cultures with 250 ml plastic flasks containing 20 ml medium with 5 % (v/v) inoculum and sealed with red rubbers. Incubations were performed in duplicate at 55 °C with shaking at 380 r.p.m. The headspace contained 5 % CO<sub>2</sub> with different concentrations of higher alkanes. Ethane and propane consumption was measured using GC and growth was observed using the spectrophotometer.

## **2.7-Chemostat cultivation**

In the chemostat reactor with methane as an electron donor, liquid volume was 550 ml, and it was operated at 55 °C with stirring at 900 rpm with a stirrer bar. The chemostat was supplied with medium at a flow rate of 14.5 ml h<sup>-1</sup> (D = 0.026 h<sup>-1</sup>), using a peristaltic pump. The cell-containing medium was removed automatically from the chemostat by a peristaltic 3 pump when the liquid level reached the sensor in the reactor. Supply of 10% CH<sub>4</sub> (v/v), 8% O<sub>2</sub> (v/v) and 68% CO<sub>2</sub> (v/v) took place by mass flow controllers through a sterile filter and sparged into the medium just above the

stirrer bar. An O<sub>2</sub> sensor in the liquid was coupled to a Biocontroller (Applikon) regulating the O<sub>2</sub> mass controller. The initial pH was 3.4 and was regulated with 1 M carbonate connected to the vessel by a peristaltic pump. The pH was gradually increased to 6.2 and after obtaining a steady state, all experiments were performed at this pH.

In the chemostat reactor with hydrogen as an electron donor, liquid volume was 1.3 L and it was operated at 55 °C with stirring at 1000 rpm. The chemostat was supplied with medium at a flow rate of 29.9 ml h<sup>-1</sup> ( $D = 0.023 \text{ h}^{-1}$ ). A gas supply of 12% H<sub>2</sub> (v/v), 10 % air (v/v) and 78% Ar/CO<sub>2</sub> (95:5, v/v) took place by mass flow controllers through a sterile filter and sparged into the medium. An O<sub>2</sub> sensor in the liquid was coupled to a Biocontroller (Applikon) regulating the O<sub>2</sub> mass controller. The initial pH was 2.9 and the pH was regulated by 1 M NaOH. A pH range from 3 to 5.5 was investigated in the steady state.

## Results

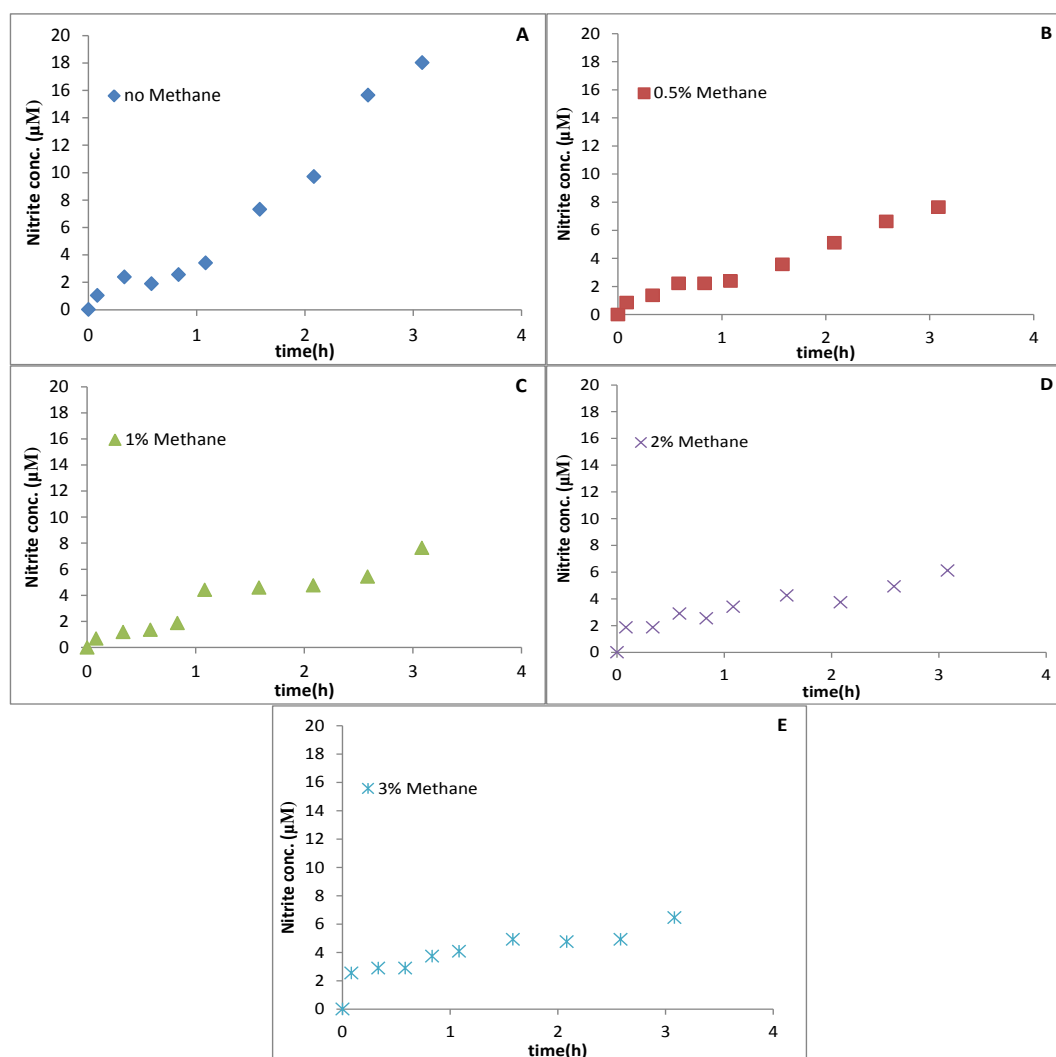
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### **3-RESULTS**

#### **3.1-Ammonium conversion to nitrite in batch experiments**

In order to study nitrification (ammonium conversion to nitrite) of the strain SolV batch incubations were performed for 3 hours. The cells were washed 3 times with medium with an r.p.m of 13000. After that the bottles were pre-incubated 45 minutes before the beginning of the experiment. After pre-incubation, different concentrations of  $\text{NH}_4^+$  were added in a range of 0.5 to 16 mM. Samples were taken every 15 minutes during the first hour and then every half an hour till 3 hours, and then placed on ice for further ammonium and nitrite determination. These procedures were the same for all the  $\text{NO}_2^-$  production experiments.

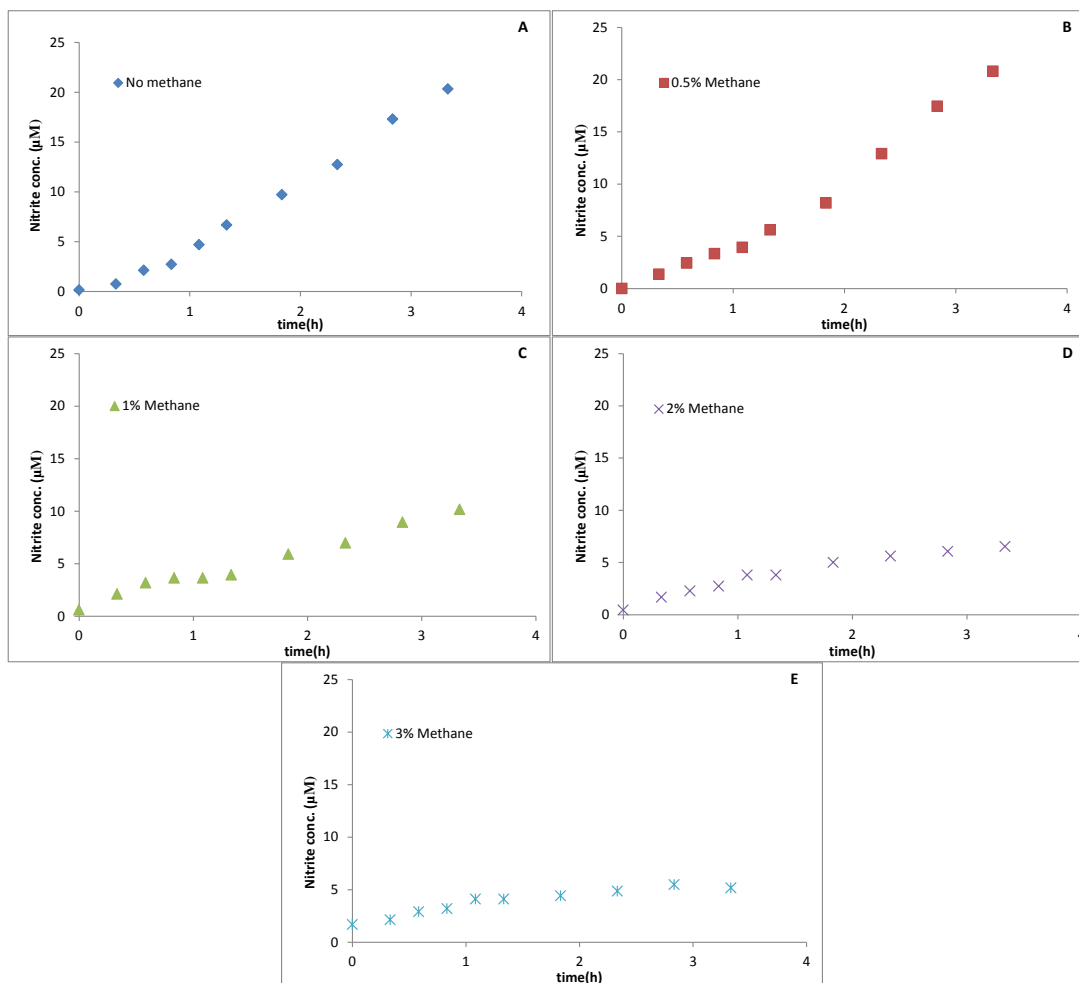
In the experiment with 0.5 mM  $\text{NH}_4^+$ , we tested 5 different incubations in duplicate with 0, 0.5, 1, 2 and 3%  $\text{CH}_4$  in each one. In the incubation without methane, after 3 hours, nitrite was produced to a final concentration of 19  $\mu\text{M}$  with a production rate of 0.039  $\mu\text{M min}^{-1}$ , which was calculated in the first hour. The final nitrite concentrations in the experiment with 0.5,1,2 and 3% methane were 9,9,8 and 7  $\mu\text{M}$ , respectively (Fig. 4).The nitrite production rates were 0.025, 0.062, 0.025 and 0.025  $\mu\text{M min}^{-1}$ , respectively.



**Figure 4** - SolV Incubations with 0.5 mM  $\text{NH}_4^+$ . (A) Incubation with no  $\text{CH}_4$ , (B) incubations with 0.5%  $\text{CH}_4$ , (C) incubations with 1%  $\text{CH}_4$ , (D) incubations with 2%  $\text{CH}_4$ , (E) incubations with 3%  $\text{CH}_4$ .

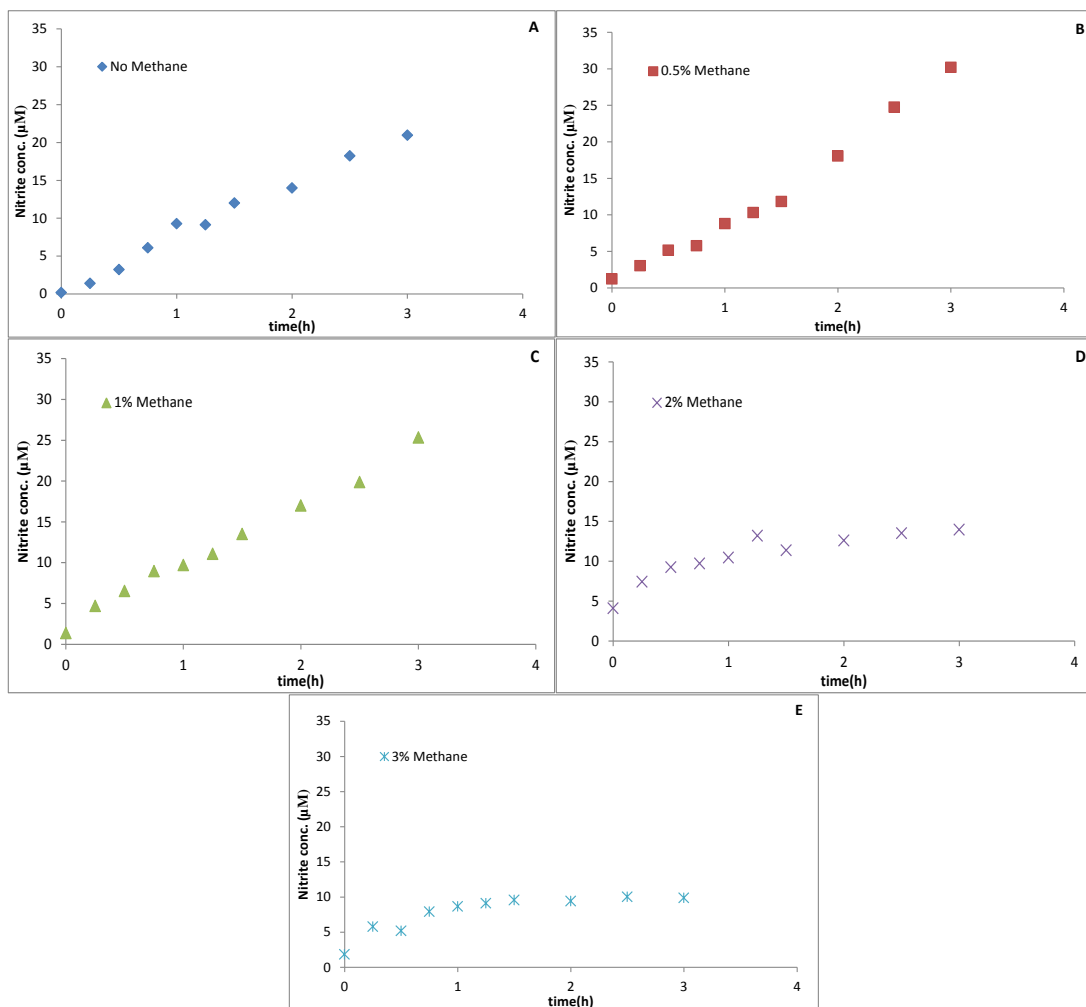
In addition to that an experiment with 1 mM  $\text{NH}_4^+$  was performed. We had 5 different incubations in duplicate within a range of 0 to 3%  $\text{CH}_4$  in each one. In the incubation without methane, after 3 hours, nitrite was produced to a final concentration of 20  $\mu\text{M}$  with a production rate of  $0.07 \mu\text{M min}^{-1}$ , which was calculated in the first hour. The final nitrite concentrations in the experiment with 0.5, 1, 2 and 3% methane were 20, 10, 7 and 6  $\mu\text{M}$ , respectively (Fig. 5). The nitrite rates were  $0.06$ ,  $0.09$ ,  $0.051$  and  $0.037 \mu\text{M min}^{-1}$ , respectively.





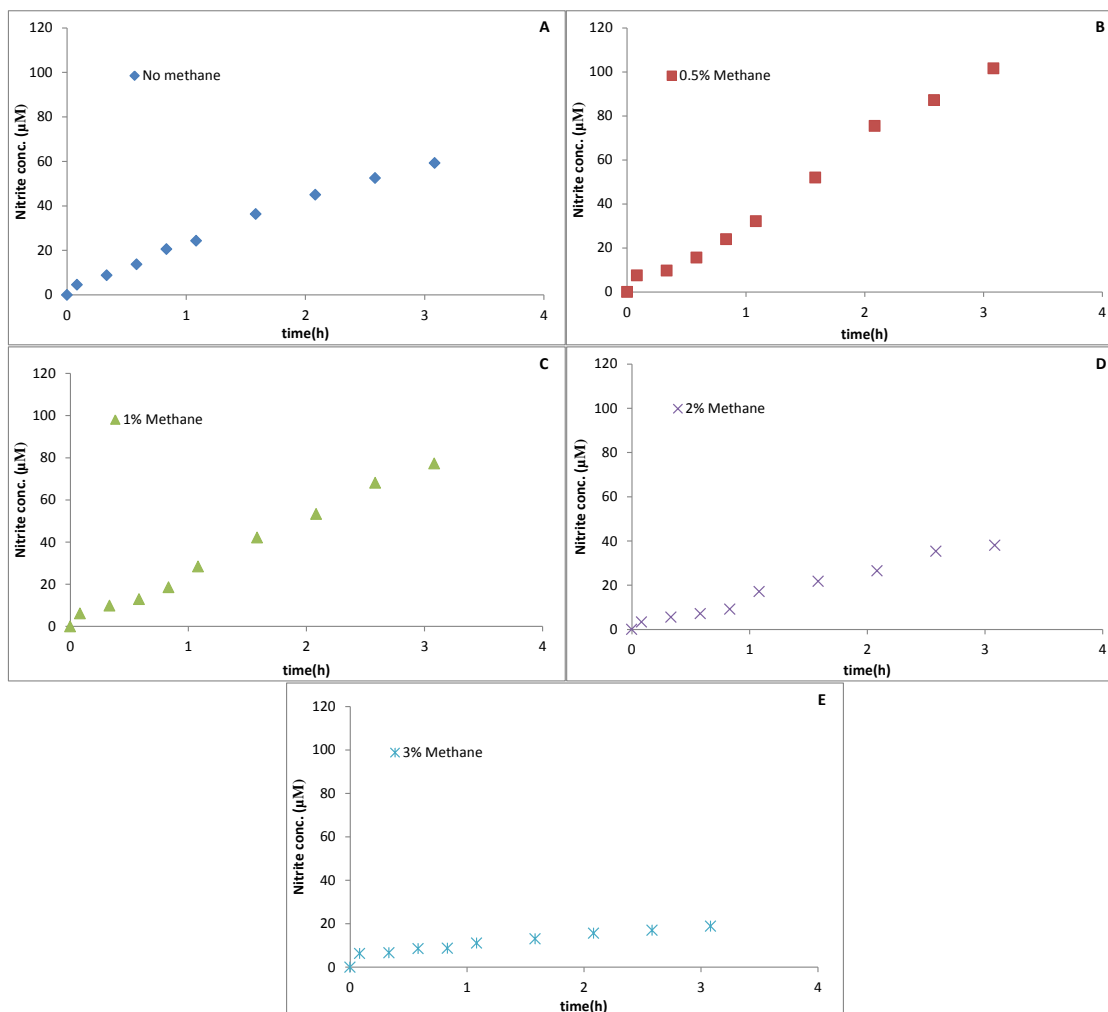
**Figure 5** - SolV Incubations with 1 mM  $\text{NH}_4^+$ . (A) Incubation with no  $\text{CH}_4$ , (B) incubations with 0.5%  $\text{CH}_4$ , (C) incubations with 1%  $\text{CH}_4$ , (D) incubations with 2%  $\text{CH}_4$ , (E) incubations with 3%  $\text{CH}_4$ .

In the experiment with 2 mM  $\text{NH}_4^+$  we had the same incubations similar to the last experiments. In the incubation without methane, after 3 hours, nitrite was produced to a final concentration of 23  $\mu\text{M}$  with a production rate of  $0.152 \mu\text{M min}^{-1}$ , which was calculated in the first hour. The final nitrite concentrations in the experiment with 0.5, 1, 2 and 3% methane were 31, 26, 15 and 10  $\mu\text{M}$ , respectively (Fig.6). The nitrite rates were  $0.126$ ,  $0.139$ ,  $0.106$  and  $0.114 \mu\text{M min}^{-1}$ , respectively.



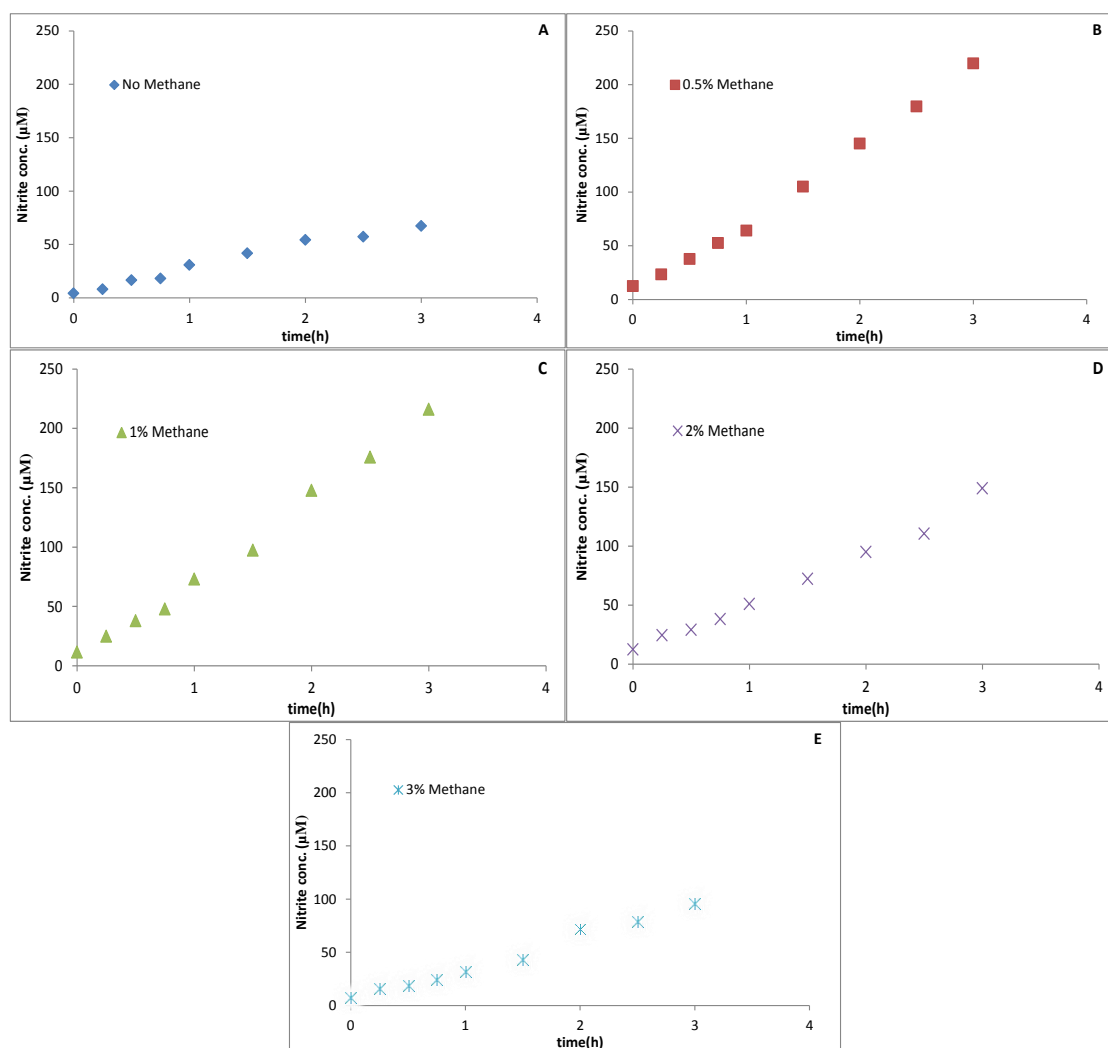
**Figure 6** - SolIV Incubations with 2 mM  $\text{NH}_4^+$ . (A) Incubation with no  $\text{CH}_4$ , (B) incubations with 0.5%  $\text{CH}_4$ , (C) incubations with 1%  $\text{CH}_4$ , (D) incubations with 2%  $\text{CH}_4$ , (E) incubations with 3%  $\text{CH}_4$ .

In the 5mM  $\text{NH}_4^+$  experiment we had the same incubations as well as the last experiments. In the incubation without methane, after 3 hours, nitrite was produced to a final concentration of 63  $\mu\text{M}$  with a production rate of  $0.329 \mu\text{M min}^{-1}$ , which was calculated in the first hour. The final nitrite concentrations in the experiment with 0.5, 1, 2 and 3% methane were 105,82,40 and 21 $\mu\text{M}$ , respectively (Fig.7). The nitrite rates were  $0.410$ ,  $0.371$ ,  $0.229$  and  $0.079 \mu\text{M min}^{-1}$ , respectively.



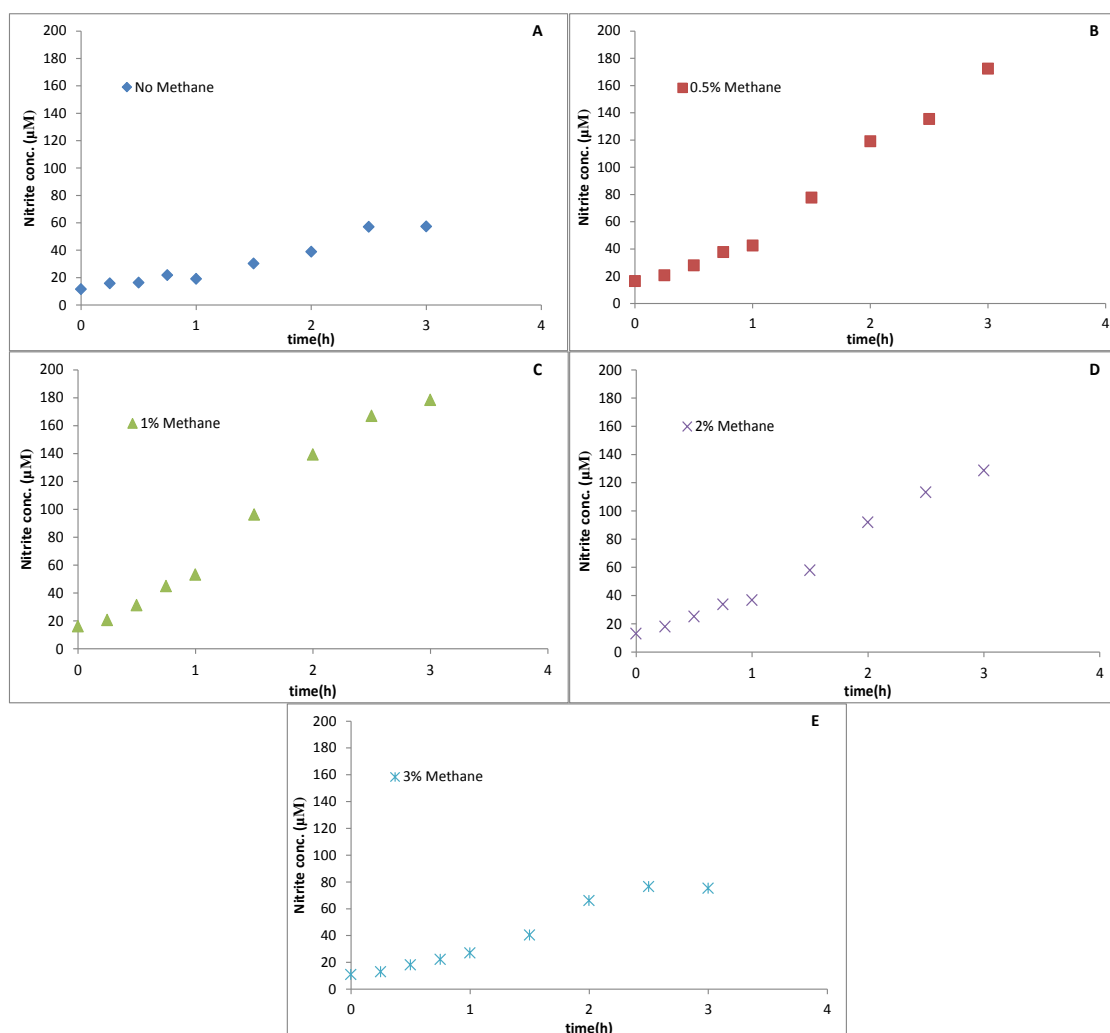
**Figure 7** - SolV Incubations with 5 mM  $\text{NH}_4^+$ . (A) Incubation with no  $\text{CH}_4$ , (B) incubations with 0.5%  $\text{CH}_4$ , (C) incubations with 1%  $\text{CH}_4$ , (D) incubations with 2%  $\text{CH}_4$ , (E) incubations with 3%  $\text{CH}_4$ .

Moreover in the experiment with 8 mM  $\text{NH}_4^+$  the procedure was identical to the last experiments. In the incubation without methane, after 3 hours, nitrite was produced to a final concentration of 70  $\mu\text{M}$  with a production rate of  $0.445 \mu\text{M min}^{-1}$ , which was calculated in the first hour. The final nitrite concentrations in the experiment with 0.5, 1, 2 and 3% methane were 240, 230, 160 and 110  $\mu\text{M}$ , respectively (Fig.8). The nitrite rates were  $0.860$ ,  $1.027$ ,  $0.643$  and  $0.415 \mu\text{M min}^{-1}$ , respectively.



**Figure 8** - SolV Incubations with 8 mM  $\text{NH}_4^+$ . (A) Incubation with no  $\text{CH}_4$ , (B) incubations with 0.5%  $\text{CH}_4$ , (C) incubations with 1%  $\text{CH}_4$ , (D) incubations with 2%  $\text{CH}_4$ , (E) incubations with 3%  $\text{CH}_4$ .

The graphs below show the 16mM  $\text{NH}_4^+$  experiment being the procedure identical to the last experiments. In the incubation without methane, after 3 hours, nitrite was produced to a final concentration of 58  $\mu\text{M}$  with a production rate of 0.126  $\mu\text{M min}^{-1}$ , which was calculated in the first hour. The final nitrite concentrations in the experiment with 0.5, 1, 2 and 3% methane were 172, 178, 128 and 75  $\mu\text{M}$ , respectively (Fig. 9). The nitrite rates were 0.435, 0.617, 0.395 and 0.268  $\mu\text{M min}^{-1}$ , respectively.

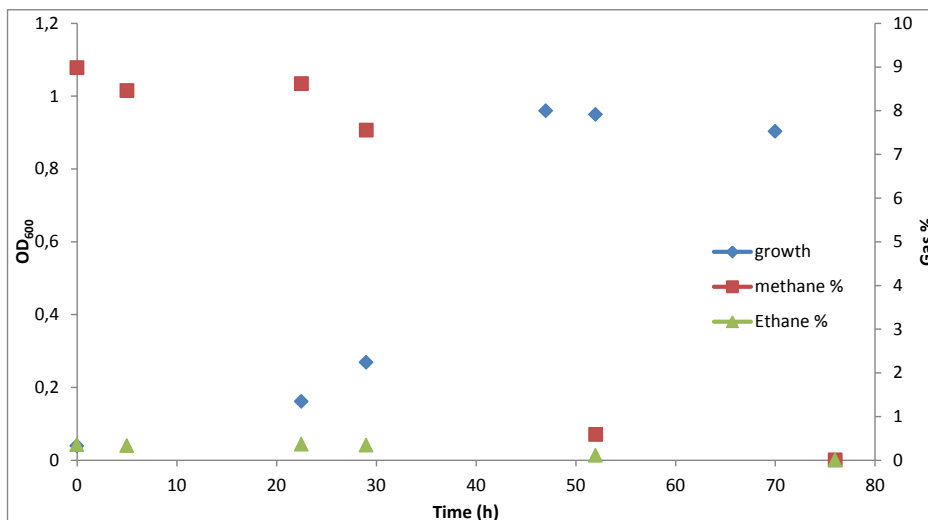


**Figure 9** - SolV Incubations with 16 mM  $\text{NH}_4^+$ . (A) Incubation with no  $\text{CH}_4$ , (B) incubations with 0.5%  $\text{CH}_4$ , (C) incubations with 1%  $\text{CH}_4$ , (D) incubations with 2%  $\text{CH}_4$ , (E) incubations with 3%  $\text{CH}_4$ .

### 3.2-Growth on higher alkanes in batch experiments

In order to study the growth of strain SolV on higher alkanes, different batch incubations were performed. The first experiment was with natural gas which contains various concentrations of different alkanes in their composition. In this experiment, we added 12 ml of natural gas that was 9% methane and 0.3% ethane of the bottle's head of space total volume. It was observed that during the exponential phase that started after 20 hours and ended after 50 hours of incubation the percentage of gases was almost

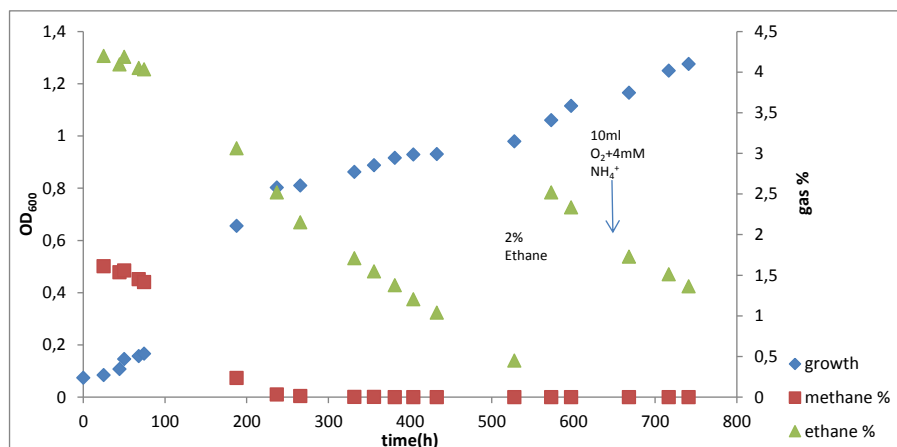
consumed. The percentage of methane went from 9 to 1.7% and ethane from 0.3 to 0%, while the OD increased till 1 (Fig.10). After observing the decrease of ethane, further experiments on higher alkanes were performed.



**Figure 10** -Incubation of SolV with natural gas containing methane and ethane at different concentrations. Observation of growth (blue squares) and methane (red squares) and ethane (green triangles) consumption.

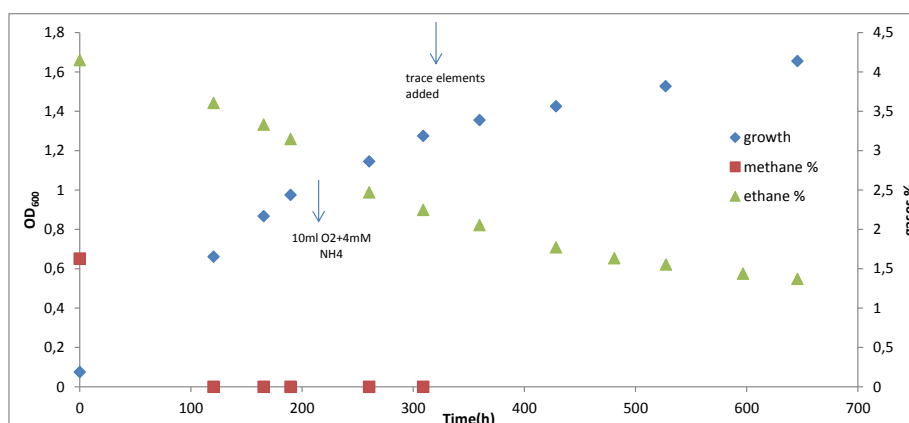
Since ethane was consumed by strain SolV, the next experiments were performed using the incubations with higher alkanes such as ethane and propane.

The incubation with ethane was performed in duplicate with 2%  $\text{CH}_4$  + 5%  $\text{C}_2\text{H}_6$  for 30 days. In this experiment the initial OD was 0.09 and it grew till 1.3. During the exponential phase that was between 90 and 200 hours it was visible a decrease in alkanes, being methane almost consumed and just remaining ethane, while the OD was approximately 0.8. Till the end of the experiment the cells grew till OD 1.3 only on ethane. During the experiment it was added to the incubation ethane,  $\text{O}_2$  (it is used in the first step of oxidation of alkanes) and  $\text{NH}_4^+$  (N-source) because they were limited (Fig.11).



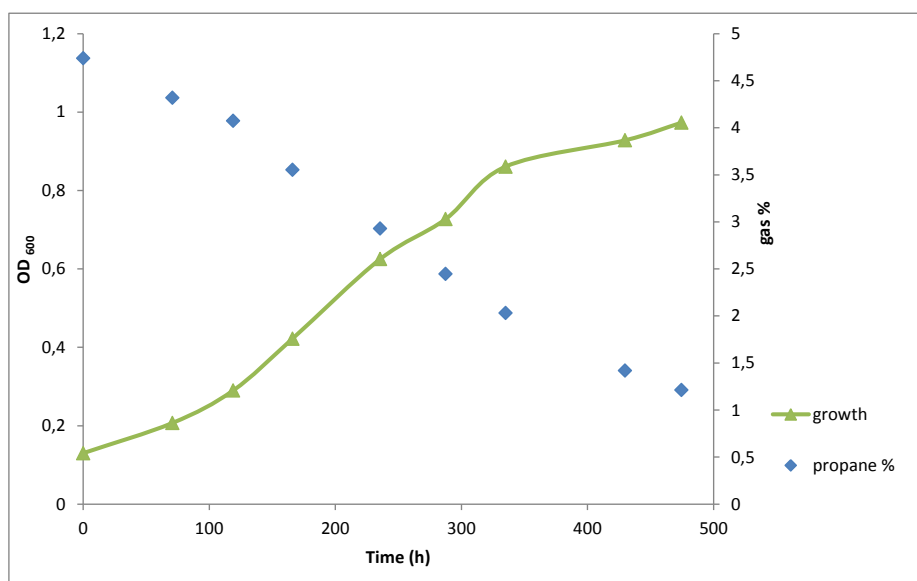
**Figure 11** - Incubation of SolV with 2% methane and 5% ethane. Observation of growth (blue squares) and methane (red squares) and ethane (green triangles) consumption.

The incubation with propane was performed in duplicate with 2%  $\text{CH}_4^+$  + 5%  $\text{C}_3\text{H}_8$  for 28 days. In this experiment the initial OD was 0.09 and it grew till 1.7. The exponential phase (which is not well visible in the graph) was between 50 and 200 hours and it was visible a decrease in alkanes, being methane completely consumed after 100 hours and just remaining propane, while the OD was approximately 1. Till the end of the experiment the cells grew till OD 1.7 only on propane. During the experiment, extra oxygen (10 ml), ammonium (4 mM) and trace elements (concentration enough till OD 10) were added because they were limited (Fig.12).



**Figure 12** - Incubation of SolV with 2% methane and 5% propane. Observation of growth (blue squares) and methane (red squares) and propane (green triangles) consumption.

After the experiment with methane and propane, other incubations were performed. In this incubation the only electron donor was propane and it was used inoculations from the previous experiment (Fig.12). The initial OD value was 0.12 and the final value was 1. The percentage used was 5% and the experience continued for 20 days. The exponential phase held between 60 and 335 hours with an OD of 1, while the percentage of propane decreased till 2%.



**Figure 13** - Incubation of SolV with 5% propane. Observation of growth (green triangles) and propane (blue squares) consumption.



## Discussion

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## 4-DISCUSSION

In this study, the nitrosative stress and growth on higher alkanes in batch experiments of *Methylophilum thermophilum* strain SolV were investigated.

Regarding nitrosative stress, nitrite production was investigated with different ammonium concentrations under methane concentration in a range of 0 to 3%.

Methane and ammonia are very similar molecules, and therefore microorganisms using ammonia as the only energy source (nitrifiers or ammonia oxidisers), and microorganisms that use methane as the only energy source (methanotrophs) possess similar features (Stein *et al.*, 2012).

The genome of SolV shows only the pMMO enzyme (particulate MMO), thus it has been hypothesized that strain SolV could oxidase ammonium to nitrite in the presence of methane.

To test this hypothesis, the experiments with different concentrations of methane and ammonium were performed. At 1% methane the nitrite production rate was found higher except with 2 and 5 mM  $\text{NH}_4^+$  experiments.

This could be because 1% methane is the ideal methane percentage limitation for the consumption of ammonium and consequently a higher nitrite production rate. A reason for those exceptions could probably be the determination reagents, because they were not fresh and these reagents lose their sensitivity over time.

Another plausible reason is the spectrophotometer, because the absorbance fluctuates from 0.002 to 0.006 which interferes with the results, because we were looking to the nM scale. Differences in the initial  $\text{OD}_{600}$  values from 0.05 to 0.1 could also interfere with this variation within the nitrite production rate.

In this experiment, it is also clear that higher concentration of ammonium leads to a higher nitrite production rate and to a higher concentration of nitrite after 3 hours of incubation.

On the other hand with 16 mM ammonium the story is different because at that value, the values of nitrite production rate and the concentration of nitrite after the 3 hours decreased. This could be because it was reached a saturation point where the enzyme cannot degrade the substrate at the same speed as at lower concentrations.

Attempts to obtain a Michaelis Menten curve was not successful, because of problems referred above, insufficient concentrations of ammonium experiments, and time intervals.

Regarding the different percentages of methane, 3 % was chosen as the maximum, because in the first attempts methane concentrations up to 10 % were tried and no nitrite production was observed.

A reason for this is because pMMO has more affinity to methane than to ammonium and with higher concentrations of methane the enzyme does not uptake the ammonium.

Afterwards, further experiments were made till 3% because 2 and 3% are the percentages where nitrite production starts to decrease in all experiments with different concentrations of ammonium.

These experiments are correlated with the rigorous review from (Hanson & Hanson, 1996) where it says that pMMO has much more affinity to methane than to ammonium in higher concentrations of methane but in low concentrations of methane the ammonium inhibit the methane oxidation.

Regarding growth on higher alkanes, SolV growth on short-chain alkanes in alternative to methane and which operon is involved was investigated.

All methanotrophs use the enzyme MMO to oxidase methane. Analyzing the genome of SolV showed that pMMO has three *pmoCAB* operons and one of these operons was not expressed under nitrogen fixing and oxygen limited conditions, where electron donor was methane (Khadem *et al.*, 2012). It is hypothesized that the third operon might be expressed under growth conditions using higher alkanes.

In order to test this hypothesis, batch incubations with natural gas were performed. The natural gas contains different gases, including alkanes, in their composition.

This experiment was continued for approximately 76 hours and it can be seen that the 0.3% and 9% of ethane and methane respectively were totally consumed after this time. Both gases were consumed during the exponential phase.

After observing the consumption of ethane even if in small concentrations the next experiments was the growth with higher concentrations and with different alkanes.

Two different experiments were initiated. The first incubation was with 2% methane and 5% ethane, and incubation with 2% methane and 5% propane.

The bottles used were of 300 ml. The percentages of gases in the graphs are a bit lower because the calculations were made for head of space of 250 ml instead of 280ml.

It was evident that on both incubations the exponential phase starts later in comparison to using higher concentrations of methane. A reason for this could be that the cells are adapting to these new alkanes.

Furthermore after the total consume of methane in both incubations it was evident that ethane and propane continued to decrease and the OD<sub>600</sub> increasing. The OD<sub>600</sub> on ethane increased till 1.3 and on propane increased till 1.7.

NH<sub>4</sub><sup>+</sup>, trace elements and O<sub>2</sub> were added in the experiments. NH<sub>4</sub><sup>+</sup> and trace elements were added to avoid any limitations. O<sub>2</sub> is needed for the first step of oxidation to be performed therefore it was added because the concentration was lower after some time.

After the consumption of different alkanes a new experiment with only propane was performed. Thus, inoculum from cells growing on methane and propane was used as cells were already adapted to this alkane. In Fig.13 is visible that SolV could grow only on propane till OD<sub>600</sub> of 1 after 10 days.

A reason for taking so long to grow could be that the cells are still adapting to grow only on propane or because they take longer to convert propane.

Experiments on ethane, ethanol, propanol and butanol were made but they need to be optimized. Nevertheless those experiments reached an OD<sub>600</sub> of 0.2/0.3.

These results correlate with the study of the methanotroph *Methylocella silvestris*, which shows that this strain could grow on propane in absence of methane (Crombie A.T. & Murrel J.C., 2014).

## Conclusions & Outlook

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## 5-CONCLUSION & OUTLOOK

In this study, we analyzed the nitrosative stress and growth on higher alkanes on “*Methylophilum fumariolicum*” strain SolV, which improves our understanding about the physiology of this bacterium.

The study of nitrosative stress shows that in strain SolV, the pMMO enzyme can use ammonium under limited values of methane and oxidize it to hydroxylamine, which further is oxidized to nitrite.

The study of growth on higher alkanes shows that this strain could also grow on propane. Regarding future work, transcriptome analysis needs to be performed to test whether the third *pmoCAB* operon is expressed under the growth on higher alkanes. Experiments with ethane, ethanol, propanol and butanol need to be optimized, and further transcriptome analysis could be performed. Experiments with butane could be also planned as well to test whether strain SolV can grow on C4 compounds.

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## **Supplementary Information**

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## 7-SUPPLEMENTARY INFORMATION

### 7.1-Appendix I

#### Nitrite Determination Protocol

**RANGE: 0.1 - 0.5 mM NO<sub>2</sub><sup>-</sup>**

#### CHEMICALS:

*NO<sub>2</sub><sup>-</sup> Reagent A:* 1% (w/v) sulfanilic acid in 1M HCl. (dissolve in the dark, dissolving takes at least half an hour)

*NO<sub>2</sub><sup>-</sup> Reagent B:* 0.1% (w/v) Naphtylethylene diaminedihydrochloride (NED)in water

#### HOW DOES IT WORK?

This assay relies on a diazotization reaction that was originally described by Griess in 1879 (Griess, 1879). In the Griess reaction nitrite reacts under acidic conditions with sulfanilic acid HO<sub>3</sub>SC<sub>6</sub>H<sub>4</sub>NH<sub>2</sub> to form a diazonium cation HO<sub>3</sub>SC<sub>6</sub>H<sub>4</sub>NN<sup>+</sup> which subsequently couples to the aromatic amine 1-naphthylamine C<sub>10</sub>H<sub>7</sub>NH<sub>2</sub> to produce a **red-violet** colored water-soluble azo dye HO<sub>3</sub>SC<sub>6</sub>H<sub>4</sub>-NN-C<sub>10</sub>H<sub>6</sub>NH<sub>2</sub>.

#### PROCEDURE

Before measuring the nitrite concentration in your sample make sure to dilute it to the above mentioned range.

1. Add 100 µl of sample to cuvette (4ml macro cuvette)
2. Add 0.9 ml milliQ (or de-ionized water)
3. Add 1ml reagent A
4. Add 1ml reagent B
5. Wait for 10 min
6. Read absorbance at 540nm

## 7. Discharge the waste appropriately

The procedure should be done with water instead of the sample for the “blank” measurement.

### **CALIBRATION AND NOTES**

Make stock solutions of 0.1 - 0.5 do the determination according to procedure.

The sensitivity can be increased 10 fold by using 1 ml sample (instead of 100 µl) and no water in step two.

### **REAGENT STOCKS AND LIQUID WASTE DISPOSAL**

Keep both reagents in dark or in amber bottles.

General stocks fitted with dispensers are kept in the analytical lab HG02.418 Reagent A is stored at room temperature in the cupboard underneath the fumehood. Reagent B is kept in door of the fridge in the same room.

Prepare new reagent stocks if the level gets below the lines indicated on the stock bottles and write down name and date of preparation.

Transfer liquid waste into the 5L nitrite determination waste vessel present in the cupboard underneath the fumehood. Empty solid waste may be disposed of in the normal waste bin. When the liquid waste vessel is full: place it in the cupboard underneath the fumehood in the chemical room HG02.414 and notify Jan.

## **7.2-Appendix II**

### **Ammonium determination protocol**

**RANGE: 0.5 – 5 mM NH<sub>4</sub><sup>+</sup>**

#### **CHEMICALS**

OPA Reagent: 0.54 % (w/v) ortho-phthaldialdehyde, 0.05 % (v/v) β-mercaptanol and 10% (v/v) ethanol in 400 mM potassium phosphate buffer (pH 7.3). Dissolve the ortho-phthaldialdehyde in ethanol before adding it to the buffer!

#### **HOW DOES IT WORK?**

β-mercaptanol creates reduced conditions. Ammonium reacts with phthaldialdehyde in reduced conditions to form a yellow fluorescent compound (an isoindole derivative) (Taylor et al., 1974).

#### **PROCEDURE**

Before measuring the ammonium concentration in your sample make sure to dilute it to the above mentioned range.

1. Add 50 µl sample (to 1.5 ml eppendorf cup)
2. Add 750 µl OPA Reagent
3. Vortex
4. Wait for 20 min in room temperature
5. Transfer to 1.5 ml cuvette
6. Read absorbance at 420nm
7. Discharge the waste appropriately

The procedure should be done with water instead of the sample for the “blank” measurement.

## **CALIBRATION**

Make stock solutions of 1 – 5 mM  $\text{NH}_4^+$ , do the determination according to procedure.

## **WASTE DISPOSAL**

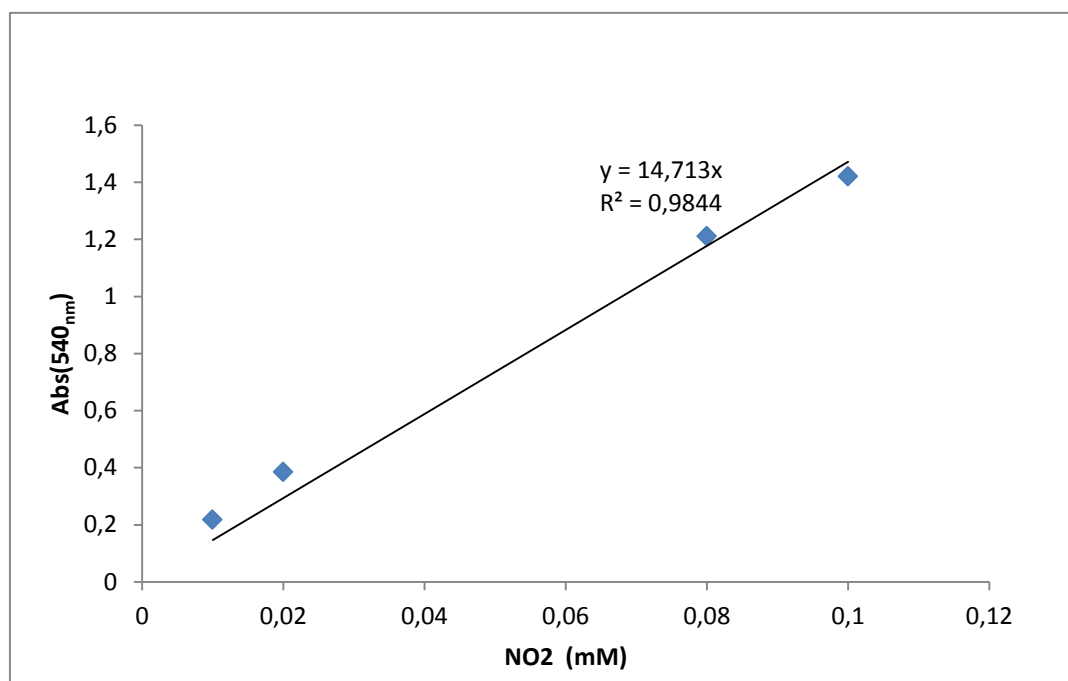
The concentration of  $\beta$ -mercaptanol is sufficiently low, and phthaldialdehyde is biologically degradable, to allow for disposal through the sink- flush down with plenty of tap water.

## **REFERENCE**

Taylor, S., Ninjoor, V., Dowd, D.M., and Tappel, A.L. (1974) Cathepsin B2 measurement by sensitive fluorometric ammonia analysis. *Anal. Biochem.* 60: 153-162.

### 7.3-Appendix III

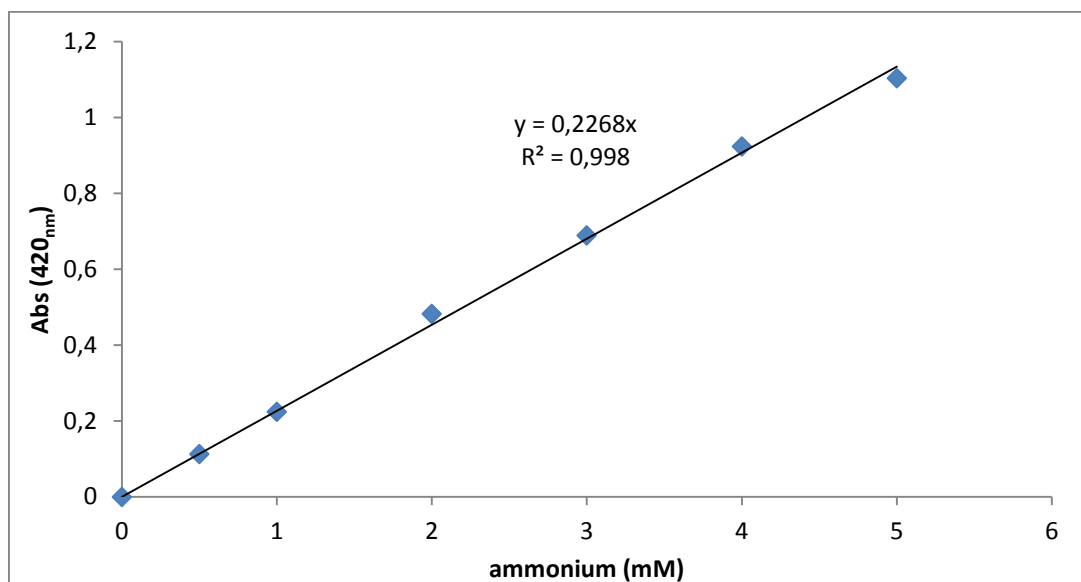
#### Nitrite standard curve



**Figure 1-** Nitrite standard curve. It was used 0.01, 0.02, 0.08, 0.1mM nitrite.

## 7.4-Appendix IV

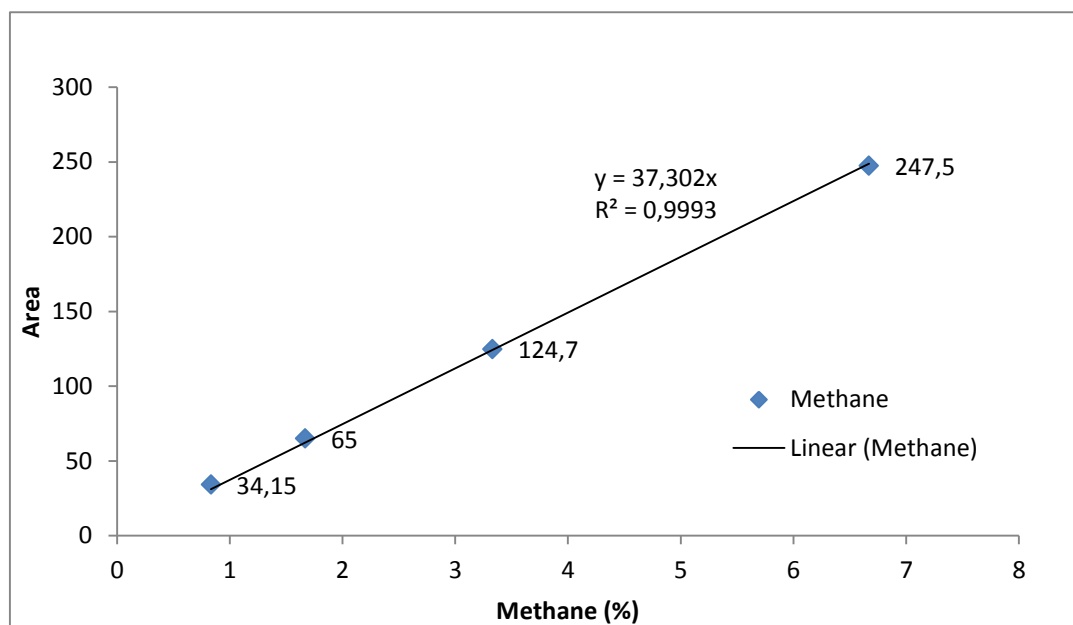
### Ammonium standard curve



**Figure 2-** Ammonium standard curve. It was used 0.5, 1, 2, 3, 4, 5mM ammonium.

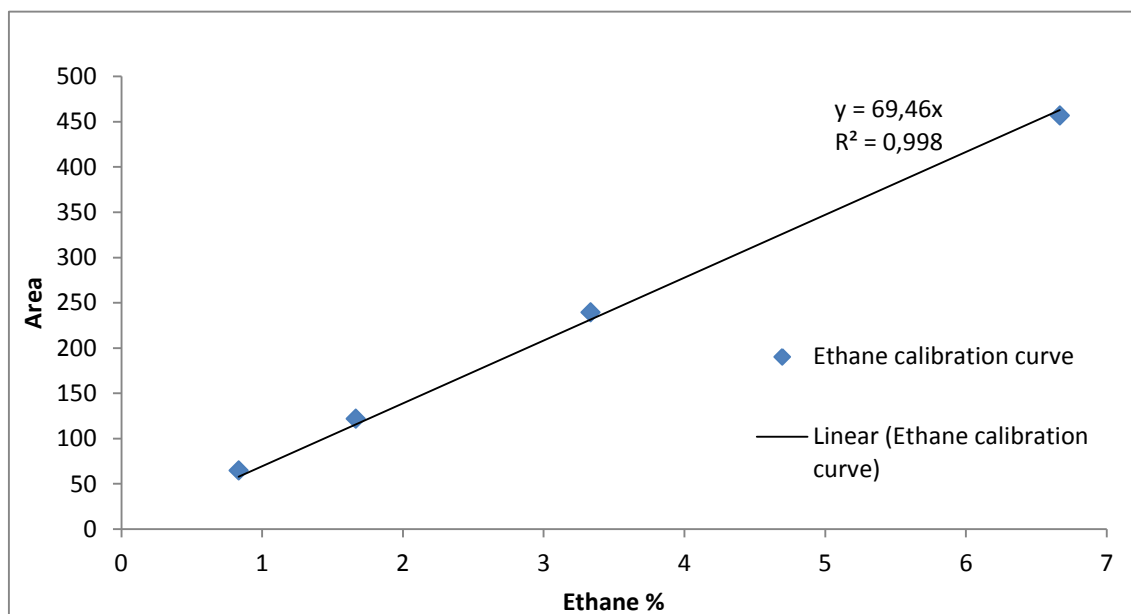
## 7.5-Appendix V

### Methane calibration curve



**Figure 3-** Methane calibration curve. It was used 0.8, 1.6, 3.3, 6.6% methane.

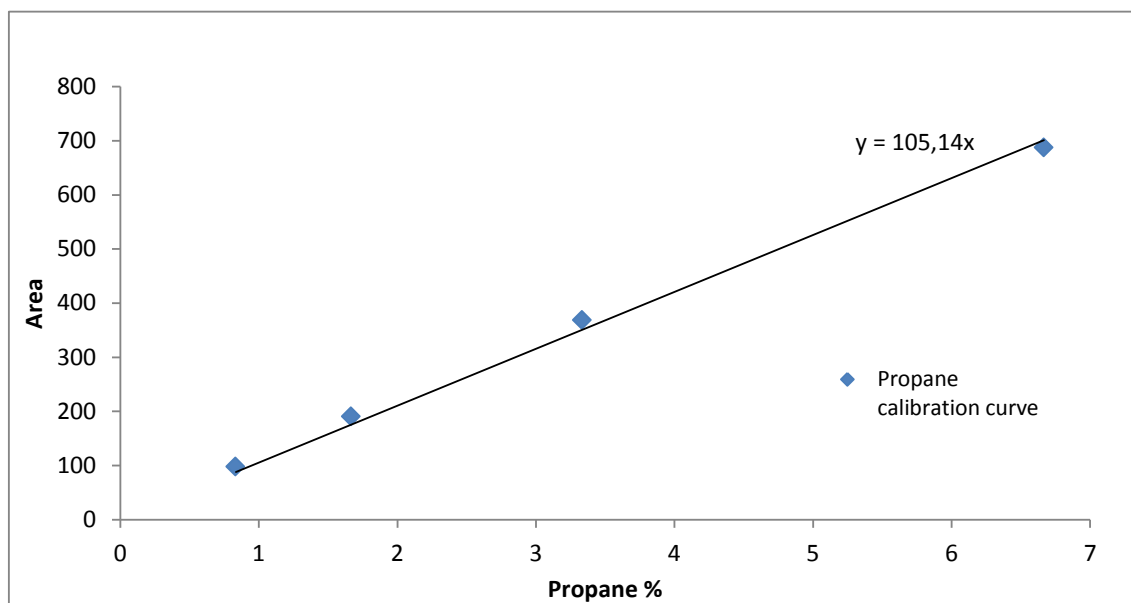
## Ethane calibration curve



**Figure 4-** Ethane calibration curve. It was used 0.8, 1.6, 3.3, 6.6% ethane.



## Propane calibration curve



**Figure 5-** Propane calibration curve. It was used 0.8, 1.6, 3.3, 6.6% propane.